

EXHIBIT E

Protein microarrays: catching the proteome

Oliver Poetz, Jochen M. Schwenk, Stefan Kramer, Dieter Stoll,
Markus F. Templin, Thomas O. Joos*

NMI Natural and Medical Sciences Institute at the University of Tübingen, Markwiesenstr. 55, 72770 Reutlingen, Germany

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Abstract

After the completion of the human genome sequencing project, DNA microarrays and sophisticated bioinformatics platforms give scientists a global view of biological systems. In today's proteome era, efforts are undertaken to adapt microarray technology in order to analyse the expression of a large number of proteins simultaneously and screen entire genomes for proteins that interact with particular factors, catalyse particular reactions, act as substrates for protein-modifying enzymes and/or as targets of autoimmune responses.

In this review, we will summarise the current stage of protein microarray technology. We will focus on the latest fields of application for the simultaneous determination of a variety of parameters from a minute amount of sample. Future challenges of this cutting-edge technology will be discussed.

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1. Introduction

Protein microarray technology has been successfully applied for the identification, quantification and functional analysis of proteins in basic and applied proteome research (MacBeath, 2002; Templin et al., 2003). These miniaturised and parallelised assay systems bear great potential as a replacement of singleplex analysis systems. The growing demands of genomics and proteomics for the analysis of gene and protein function in a global perspective has increased interest in microarray-based assays enormously. The basic principles of miniaturised and parallelised ligand binding assays were already described in the early 1980s by Roger Ekins' ambient analyte theory (Ekins, 1989). Today, DNA microarrays are well-established high-throughput hybridisation systems that enable the exploration of the whole transcriptome in a single experiment (Arora, 2002). However, there is no absolute correlation between mRNA expression levels and corresponding protein expression (Gygi et al., 1999). Furthermore, it is impossible to deduce the functional state of a protein purely from its expression

level. Therefore, additional high-throughput technologies are required that will facilitate the analysis of the function of the proteome. Within the last few years, microarray technology has expanded beyond DNA chips. A large variety of protein microarray-based approaches have already demonstrated that this technology is capable of filling the gap between genomics and proteomics.

Depending on the field of application, protein microarrays can be classified into two categories: (1) Arrays for proteomics or focused protein profiling and (2) arrays for functional studies. The first category can be subdivided by array format into forward- and reverse-phase protein microarrays (Fig. 1). The difference between the two refers to the way the sample is applied. On forward-phase protein arrays, the sample is incubated on the array so that different analytes can be detected simultaneously. Examples include antibody microarrays that are used for the identification and quantification of target proteins. Reverse-phase arrays are the latest protein microarray developments. The array consists of different samples that are immobilised on a chip. In a single step, a large collection of probes can be screened for the presence or absence of one distinct target protein (Bouwman et al., 2003; Kononen et al., 1998; Paweletz et al., 2001; Petricoin and Liotta, 2002; Yan et al., 2003).

* Corresponding author.

E-mail address: joos@nmi.de (T.O. Joos).

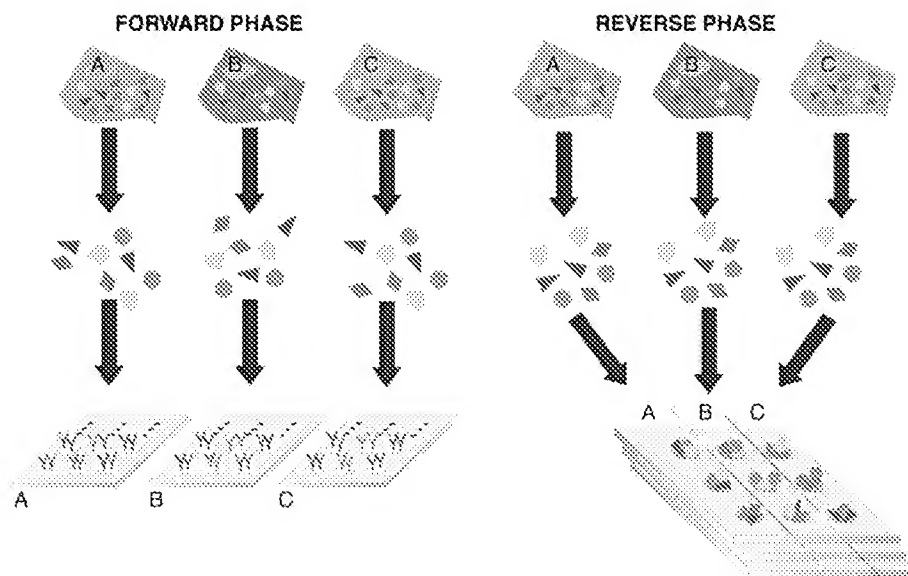


Fig. 1. Forward and reverse-phase protein capture assays. In the forward array format, each protein mixture derived from a different source is analysed on one microarray containing several hundreds or thousands capture molecules. The analyte is detected either by direct labelling or a second specific antibody. A wide range of proteins can be analysed in one sample in one experiment. The array of the reverse approach contains hundreds of protein samples immobilised on the chip. One protein is assayed in a large number of patient or cell samples. Furthermore, the sample can be fractionated in order to enhance sensitivity. Hundreds of these arrays can be generated with a tiny amount of sample and probed with hundreds of antibodies.

Apart from planar microarrays, there are also microsphere (bead)-based systems, which provide an excellent alternative for measuring a low number of analytes in a multiplex fashion (Fig. 2). Different colour- or size-coded beads are

used as solid support on which capture molecules are immobilised. Flow cytometry systems enable the simultaneous discrimination of bead types and the quantification of an analyte with suitable reporter dyes. Moreover, protein

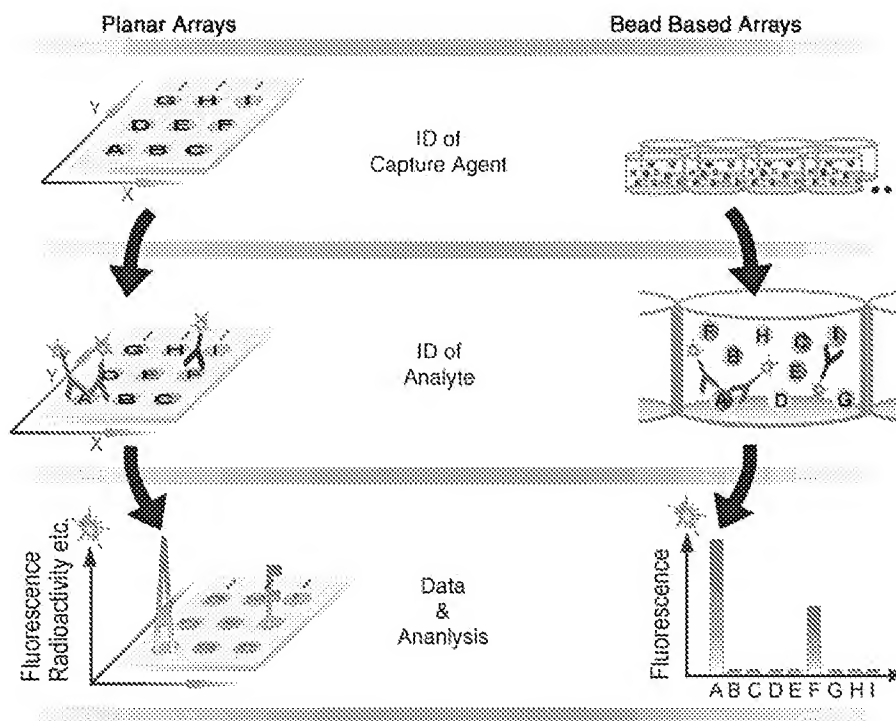


Fig. 2. Planar or bead-based array. Both platforms can be applied to multiplex analysis. A planar microarray can contain hundreds and thousands of different capture molecules whereas the bead-based system is limited to the number of available microspheres. In the planar system the signal of the binding molecule can easily be allocated to a xy position on the support. The bead-based system uses dye- or size-coded microspheres. Planar arrays rely on chemoluminescence, radioactivity, mass spectrometry or fluorescence as the read-out systems. The bead-based system applies preferentially fluorescence detection.

microarray technology enables functional protein analysis in high multiplicity. The interaction of proteins with hundreds to thousands of immobilised molecules such as proteins, peptides, low molecular weight compounds, oligosaccharides or DNA has already been shown (MacBeath, 2002; Templin et al., 2003).

Highly specific and selective capture molecules are an absolute prerequisite for the design and generation of protein and DNA microarrays. These requirements are met for DNA. DNA is a uniform molecule and highly specific capture molecules are easily designed according to the rules of Watson–Crick base-pairing. High-throughput oligonucleotide synthesis or PCR-based approaches enable a fast and inexpensive production of DNA capture agents. Therefore, the set-up of DNA arrays and the design of simple assays are straightforward. The protein world is more complex due to the structural differences between DNA and proteins. The side chains of the common 20 aminoacids provide much more possibilities for interactions than the one-by-one pairing of the four nucleobases. Proteins contain several structural elements like helices or β -sheets, they can be composed of several subunits and they can be organised in multi-protein complexes. Dynamic post-translational modifications, such as glycosylation or phosphorylation have an additional influence on protein interactions and activity. To retain their functionality, they have to be tethered

to a support without damaging their structure. Keeping immobilised proteins functionally active is much more difficult than it is for immobilised oligonucleotides or PCR fragments. Further it is impossible to predict high-affinity protein capture molecules on the basis of an amino acid sequence. Cost-effective methods must be developed that enhance the speed and throughput of protein expression. All those facts have to be considered when generating protein microarrays and performing protein microarray assays. However, the first obstacles have been overcome and a broad knowledge on how to immobilise proteins and preserve their functionality is available. These achievements have already led to successful applications of protein microarray technology in a large variety of assay systems.

In the following sections, the different protein microarray assays and their applications in protein profiling and interaction studies will be discussed.

2. Arrays for proteomics and focused protein profiling

2.1. Arrays for proteomics—antibody arrays and direct labelling

Similar to the dual-colour labelling approach used for the visualisation of differential mRNA expression, the

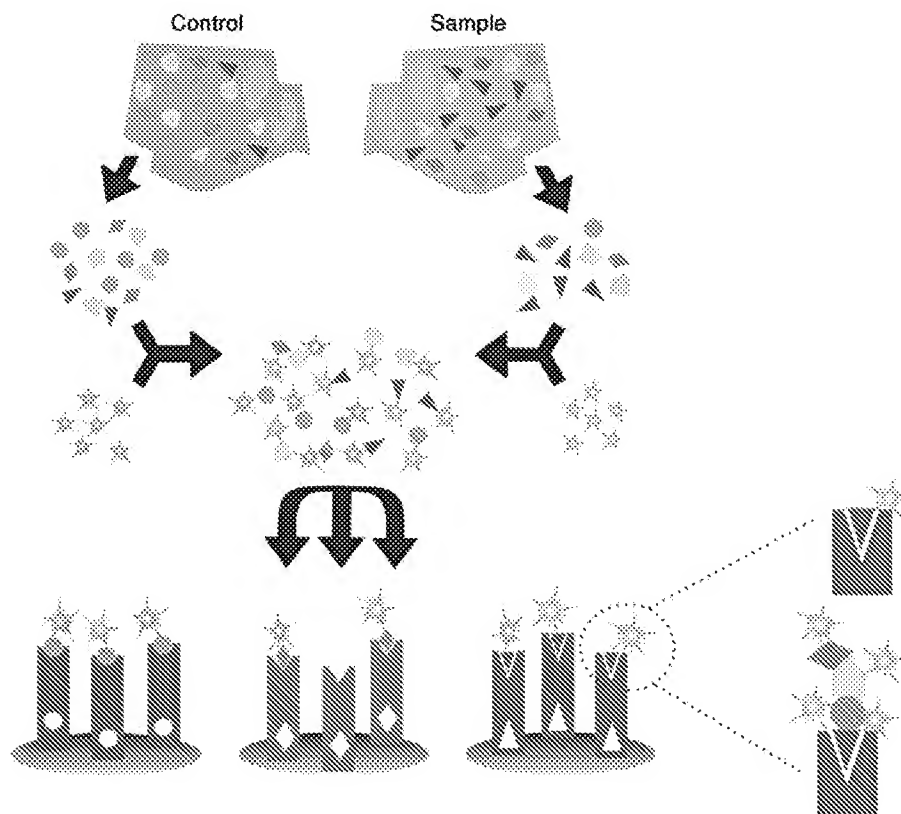


Fig. 3. Microarrays for differential protein display. Proteins from controls and samples are isolated and conjugated to distinct fluorescent molecules. The samples are mixed in equal amounts and incubated simultaneously on an antibody microarray. Subsequently, target molecules will be captured by their specific antibody. The differences in protein expression are directly reflected by the overlay of the colour signal. The enlarged section (dotted circle) indicates the difficulty of quantification. A prominent signal could be the result of a single protein but also of a large protein complex.

feasibility to differentially display proteins has already been demonstrated (Fig. 3). Haab et al. (2001) first introduced the antibody antigen microarray assay. Protein samples from two different sources were labelled, each with a different fluorophore. Equal amounts of total protein were mixed and incubated on the antibody microarray. The differences in the concentrations of the target proteins in each capture spot were detected via dual wavelength fluorescence analysis using a standard biochip scanner. However, care must be taken when data obtained from such an assay are analysed. Since proteins often assemble in multi-protein complexes, a strong signal can either result from a large amount of target protein or from a huge complex of different proteins bound to the captured target. Nevertheless, several groups have already proved the general applicability of this approach. Sreekumar et al. (2001) demonstrated the use of antibody microarrays for the detection of altered protein levels of LoVo Colon carcinoma cells after treatment with ionising radiation. Knezevic et al. (2001) were able to show cancer-specific alterations in protein levels in specific cellular populations from squamous cell carcinoma of the oral cavity. Using antibody microarrays consisting of several hundreds of antibodies, both groups identified more than ten proteins altered in expression levels in response to radiation treatment or in correlation to tumour progression. A modified approach was performed by Ivanov et al. (2004). For the multiplex detection of posttranslational modifications, Ivanov and colleagues immunoprecipitated proteins with antibodies against ubiquitinylation, acetylation and phosphorylation. The precipitates were labelled and incubated on an antibody array. The authors profiled protein tyrosine phosphorylation, ubiquitinylation and acetylation in cells that were treated differently.

An alternative antibody array based approach was developed by Belov et al. (2001, 2003). This approach involves the analysis of CD (cluster of differentiation) antigen expression on different leukaemia cells. Microarrays composed of up to 90 different anti-CD antibodies were incubated with a cell suspension. The cells, which expressed the corresponding CD antigen, bound to their immobilised capture antibody. Whole cells were used as read-out system. Different cell binding patterns were obtained for normal peripheral blood leukocytes and different types of leukaemia. These types of cell-capture antibody microarrays do not require fluorescent tags or other sophisticated detection systems. They might be an interesting approach to characterise different types of leukaemia and complement chip-based mRNA-expression profiling or flow cytometry assays. In addition, these antibody array-based approaches enable the characterisation of captured cells with additional fluorescence-labelled antibodies. In a similar approach, Soen et al. (2003) recently demonstrated the use of microarrays containing peptide–MHC (major histocompatibility complex) complexes for the identification and characterisation of multiple antigen-specific T-cell populations. Cytotoxic ($CD8^+$; CTL) or helper ($CD4^+$) T-cells were

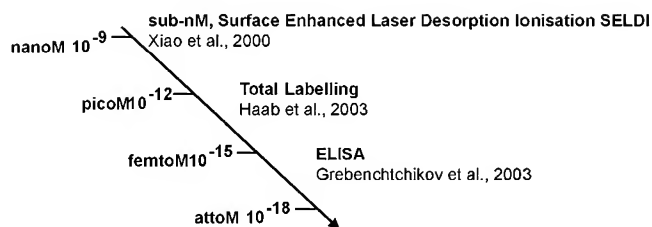


Fig. 4. Sensitivity of analytical methods for protein quantification.

captured by the arrays according to their MHC-peptide complex specificity. The authors demonstrated that it was possible to detect a rare population of antigen-specific T-cells after vaccination. The chance of obtaining antigenic epitopes during an immune response might render this approach very useful in experiments focusing on vaccine optimisation.

Antibody array approaches certainly have advantages and disadvantages. In principle, the direct labelling approach has the potential to capture thousands of proteins using high-density antibody microarrays. However, this approach is currently limited by the lack of highly specific capture reagents. Furthermore, the current assay is not sensitive enough to measure low abundance proteins. Moreover, the chemical labelling of the proteins can destroy epitopes by covalent coupling of dyes or haptens. Similar to DNA-chip technology's competition with highly sensitive RT-PCR, array-based proteomic approaches have to compete with most sensitive ELISA techniques (Fig. 4). As long as only a limited number of parameters need to be analysed, miniaturised and multiplexed immunoassays can be regarded as an excellent solution for focused protein profiling approaches. In contrast to high-density DNA chips, which definitely facilitate a global view into the transcriptome, the current antibody microarrays only permit the expression analysis of selected target proteins. As long as the protein microarray content is limiting, array-based proteomics has difficulties in becoming a sincere alternative to classical methods. Nevertheless the first antibody microarrays consisting of several hundred antibodies have already become commercially available.

2.2. Focused protein profiling—multiplex sandwich immunoassays

Focusing on a limited number of analytes, multiplex sandwich immunoassays offer an alternative to measure proteins in parallel. The use of two antibodies in a sandwich ELISA enhances specificity and sensitivity. This allows the determination of low abundance proteins down to the femtomolar range (Grebentchikov et al., 2004). Such an approach, however, requires two specific antibodies for each protein. This again increases the need for larger numbers of specific capture molecules and for methods to characterise them. The miniaturisation of sandwich immunoassays was already demonstrated in the past (Bellisario et al., 2001;

Carson and Vignali, 1999; De Jager et al., 2003; Dunbar et al., 2003; Finckh et al., 1998; Knight et al., 2004; McBride et al., 2003a; McBride et al., 2003b; Nielsen et al., 2003; Prabhakar et al., 2002; Schweitzer et al., 2002; Shao et al., 2003). One of the very first microarray-based analytical assays was developed by Ekins and colleagues (Ekins, 1989; Finckh et al., 1998). Using a fully automated microspotting system, they were able to quantify analytes such as thyroid stimulating hormone (TSH) or hepatitis-B-surface antigen (HbsAG) accurately down to the femtomolar concentration range. Shao et al. have published the most complex multiplexed sandwich immunoassay study so far. This assay allowed the quantification of 150 different cytokines and other serum components. The assay was carried out on one slide on 16 subarrays (Shao et al., 2003). The rolling-circle amplification (RCA) technology was applied for the highly sensitive detection of bound analytes. More than 50% of these miniaturised sandwich immunoassays exhibit detection sensitivities in pg/ml ranges. The same group also demonstrated that the RCA detection principle was suitable for the detection of single binding events on microspots (Schweitzer et al., 2000). Knight et al. demonstrated the suitability of multiplex cytokine assays for the profiling of 16 cytokines in human blood (Knight et al., 2004). The results of the microarray-based sandwich immunoassay were compared with results gained with established and approved ELISA assays. Identical cytokine concentrations could be determined with either method in endotoxin-stimulated human blood. Besides the investigation of serum markers, the measurement of cell proteins and their alterations are of even greater interest. The complex networks of cell-signalling systems define the need for parallel quantification of relevant proteins at different phosphorylation states. Nielsen et al. (Nielsen et al., 2003) described an antibody microarray integrated in 96-well microtiter plates with which they could quantify amount and phosphorylation state of ErbB receptors in cell lysates. Using a two-colour detection system, the total amount of the receptor and the phosphorylated form of the ErbB receptor could be measured simultaneously. Different cell lines showed significant differences in ErbB expression and ErbB phosphorylation in response to EGF stimulation.

Sandwich immunoassays on planar supports are suitable for measuring several dozens of analytes in a single experiment. However, planar array approaches still lack the power of full automation. It is still a cumbersome task to screen a large number of samples. Bead-based systems are an interesting alternative to planar microarrays; in particular when the number of components to be determined in parallel is rather small (Fig. 2). Sensitivity, reliability, and accuracy of these systems are similar to those observed with well-established ELISA procedures. A set of up to a hundred different colour-coded beads for miniaturised multiplexed ligand-binding assays is commercially available. Bead-based array systems have been used to measure the levels of cytokines or antibodies in biological samples such as patient

serum or cell culture supernatant (Bellisario et al., 2001; Carson and Vignali, 1999; Chen et al., 1999; De Jager et al., 2003; Dunbar et al., 2003; Fulton et al., 1997; McBride et al., 2003a,b). Opalka et al. (2003) presented a method for the detection of neutralising antibodies against four types of human papilloma viruses. Using a competitive immunoassay, the titers of neutralising antibodies could be measured in serum with the same sensitivity and accuracy as a competitive radioimmunoassay. McBride et al. (2003a) have developed a bead-based multiplexed sandwich immunoassay to detect a broad range of pathogens including virus, protein toxins and bacterial spores. The multiplex assay complied with the requirements of a rapid, robust and sensitive detection method and was comparable with standard ELISA techniques. The same group of researchers constructed a fully autonomous unit for monitoring continuously airborne biological threat agents (McBride et al., 2003b) and demonstrated that the system successfully detected *Bacillus anthracis* and *Yersinia pestis* strains.

The increasing number of commercially available assay kits has provided the bead-based systems with the great potential to become an established tool for multiplexed assays. Several companies offer bead-based sandwich immunoassays for the detection of cytokines, metabolic markers, cell signalling molecules, phosphorylated and unphosphorylated kinases etc. in parallel.

2.3. Focused protein profiling—reverse-phase microarrays

An alternative microarray format is the reverse screening approach. With this method, tiny amounts of a tissue or cell sample are immobilised in a microarray format onto a solid support (Jones et al., 2002; Paweletz et al., 2001). Tissue or cell lysates are prepared from differently treated cultured cells or from microdissected tissues (e.g., procured by laser capture microdissection (LCM)), followed by the generation of microarrays. The immobilised protein mixtures represent the whole repertoire of cellular proteins at a certain state. These microarrays are then screened for the presence or absence of specific target proteins using highly specific and selective antibodies. This set-up allows the screening of a large collection of tissue or cell lysates with a large number of antibodies or patient sera. Overall, only very low amounts of sample are required.

A well-designed, reverse-phase approach to analysing protein expression within 60 different human cancer cell lines (NCI-60) was recently demonstrated by Nishizuka et al. (2003). Each cell line was printed in 10 two-fold serial dilutions. Monoclonal antibodies directed against 52 different target proteins were used. Signals were clustered and protein expression patterns obtained. The researchers identified two promising pathological markers that distinguish colon from ovarian adenocarcinomas. The detailed comparison of protein expression with mRNA expression data from DNA microarrays revealed a high correlation concerning cell-structure-related proteins across all NCI-60

cell lines. Non-cell-structure-related proteins exhibited no correlation between the amount of mRNA and the level of protein.

Reverse-phase protein arrays represent an interesting technology used to study the fluctuating state of the proteome in minute cell quantities. The activation status of cell signalling pathways controls cellular fate. Deregulation of these pathways can result in carcinogenesis. Changes in pathway activation that occur between early stage prostatic epithelial lesions, prostatic stroma and the extracellular matrix can be analysed by obtaining pure populations of cell types by laser capture microdissection (LCM) and analysing the relative states of several key phosphorylation points within the cellular circuitry. Grubb et al. (2003) have applied reverse-phase protein array technology in order to analyse the status of key points in cell signalling involved in pro-survival, mitogenic, apoptotic and growth regulation pathways in the progression from normal prostate epithelium to invasive prostate cancer. Focused analysis of phospho-specific target proteins revealed changes in cellular signalling events through disease progression and between patients. Gene expression alone cannot determine the activation (e.g. phosphorylation) state of *in vivo* signal pathway checkpoints. Aberrations in the regulation of these pathways may be a key to carcinogenesis. Cell culture systems and animal models may not accurately reflect these changes (Ornstein et al., 2000). Reverse-phase protein microarrays allow the examination of the relative states of several key phosphorylation checkpoints in pathways involved in pro-survival, mitogenic, apoptotic and growth regulation pathways involved in the progression from normal prostate epithelium to invasive prostate cancer.

A drawback of the reverse-phase approach is the low concentration of target molecules of interest. High abundance proteins can easily be detected in a protein mixture, whereas rare protein species, i.e. proteins such as signalling molecules or kinases, are only present in low concentrations in a microspot. As demonstrated with Western blot technology, the separation and concentration of proteins prior to detection by specific antibodies greatly enhances detection sensitivity, even for low abundance proteins. In the expanding field of proteomics, it is important to be able to separate complex protein mixtures with increasing demands on sensitivity, automation and reproducibility. Since its introduction in the 1970s, two-D gel electrophoresis has become the technique of choice for proteome analysis for the separation of proteins or peptides according to their *pI* and mass (Pandey and Mann, 2000). The pre-fractionation of crude protein extracts prior to the generation of reverse microarrays is one way to increase sensitivity. This allows a set-up in order to screen for rare biomarkers. The group of Samir Hanash has shown the benefits of pre-fractionation of cell lysates by chromatography prior to array generation (Bouwman et al., 2003; Nam et al., 2003). Solubilised proteins from the LoVo colon adenocarcinoma cell line were separated into 1760 fractions

according to their *pI* and molecular weight. The same was done for the prostate cancer cell line LNCaP. Subsequently, these fractions were arrayed onto nitrocellulose-coated slides and screened with the sera of cancer patients. Several types of autoantibodies were detected. A distinct pattern of reactivity was observed with sera derived from patients with colon cancer relative to patients with lung cancer diagnosis. This approach allowed the identification of autoantigens by further analysis of the fraction of interest using standard mass spectrometric approaches. Finally, using reverse-phase protein microarrays, it is possible to screen a large number of patient samples for the presence of autoantibodies directed against tumour proteins. Two major advantages of reverse microarray screening are that the sample proteins do not need to be labelled and that only one single antibody is required. The reverse screening approach hence provides a new approach to identify biomarkers and could be useful in the early profiling of drug candidates with regard to their efficacy and toxicity.

Another reverse screening technology is the surface enhanced laser desorption ionisation (SELDI) approach (Caputo et al., 2003; Petricoin et al., 2004; Petricoin and Liotta, 2002; Tang et al., 2004). Cell extracts derived from different sources or serum samples are incubated on different macro spots of the same adsorptive surface chemistry (e.g., pseudo-specific chromatographic surfaces like cation/anion exchange material or hydrophobic surfaces, immobilised-metal affinity surfaces, and biospecific interactive surfaces). Unbound or weakly bound proteins can be washed away, thus reducing the complexity of the sample. The whole variety of non-specifically captured target proteins is analysed by mass spectrometry. The mass spectrum shows the different molecular weights of the captured proteins. The comparison of two data sets generated from two different samples immediately identifies differentially expressed proteins by the ratio of intensities. In some cases, the differentially displayed proteins can be identified immediately on the basis of their molecular weights. However, in most cases it is necessary to identify such markers separately. This can be achieved easily using the same adsorptive material as used for the SELDI experiment after protein enrichment by affinity chromatography. The enriched proteins can then be identified by standard methods (e.g. Edman sequencing, western blot, digest mass fingerprinting). The SELDI technology is easy to handle and suitable for the rapid detection of differences in total protein content of different samples. As the detector sensitivity of time-of-flight mass analysers decreases with increasing molecular weights, SELDI is perfectly suited for the detection of small proteins and peptides. However, it exhibits limitations with respect to high-molecular weight proteins or membrane proteins. Although assay sensitivity in SELDI experiments is much lower in comparison to sandwich immunoassays, the SELDI approach is still a rapid screening platform for any unknown protein biomarker (Bandera et al., 2003; Uchida et al., 2002; Zhang et al., 2002).

Another interesting reverse-phase approach was recently published by Wang (2004). An antibody array-based immunostaining method was developed which allows the simultaneous detection of a large number of different proteins in one immobilised cell type. A 'dissociable' antibody array was generated and brought into close contact with a cell lawn to be screened for the presence of the appropriate antigens. Cells were cultured on a support onto which the antibody array was placed. The antibodies dissociated from the array support and bound to the cellular antigens if present. These antibodies were then visualised by standard immunohistochemistry techniques. Such 'dissociable' antibody arrays can be used to analyse the expression and the subcellular localisation of different proteins in various cultured cell types.

Currently, tissue microarrays represent the most prominent reverse screening approach (Fig. 1). Microarrays of tissue samples containing hundreds of tissue specimens can be screened for the presence or absence of DNA or RNA molecules or proteins (e.g., p53 or tumour markers Her-2 and EGFR, etc.). Standard analytical methods such as immunohistochemistry, fluorescence in situ hybridisation (FISH) or other molecular detection methods can be used for detection (Chung et al., 2004; Freier et al., 2003; Kang et al., 2003; Kononen et al., 1998). Tissue microarrays have a considerable advantage over conventional histological approaches: a large number of specimens can be treated simultaneously in an identical manner. The traditional histological analysis of tissue specimens is still a rather slow and labour-intensive process. In contrast, the parallel processing of a large number of histological samples increases the throughput dramatically. Therefore, tissue microarrays are the method of choice for screening a large number of samples for well-defined proteins. However, one disadvantage of tissue microarray analysis might remain. It is difficult to determine whether biopsy material represents the whole specimen since it only represents a tiny fraction of the whole tumour (see discussion in (Sauter et al., 2003)).

3. Protein microarrays for functional studies—protein interaction analysis

Conventional biochemical methods analyse protein–protein interactions one by one. The yeast-two-hybrid system has generated a tremendous amount of protein interaction data. The generated data are integrated into interaction maps of high complexity (Uetz et al., 2000). Additional to this approach, a method based on a two-step affinity precipitation coupled to mass spectrometry was developed by Rigaut et al. (1999) in order to explore the composition of protein complexes. Two hundred and thirty two protein complexes could be defined with 1739 yeast protein constructs (Gavin et al., 2002). Protein microarray technology represents a complementary method for the study of protein interaction in vitro. So

far, the analysis of protein–protein, enzyme–substrate, protein–DNA, protein–oligosaccharide and protein–drug interactions has been described. Low and high density arrays of proteins, peptides and small molecules have been used to study the binding of DNA, RNA, small chemical ligands and proteins to their immobilised binding partners (Burns-Hamuro et al., 2003; Ge, 2000; Houseman et al., 2002; Lizcano et al., 2002; Reineke et al., 2001; Zhu et al., 2000, 2001).

To date, one high-density protein microarray-based approach using a yeast proteome chip containing 5800 different recombinant yeast proteins was used to study protein interaction (Zhu et al., 2001). Protein–protein interactions of the yeast proteome were tested with biotinylated calmodulin. Six out of twelve already well-known interactions were confirmed and additional 33 new potential binding partners were identified. Based on these results, a potential common binding motif of calmodulin was determined. These proteome arrays were also tested with phospholipids, and 150 putative phospholipid-binding proteins could be detected. In addition, the yeast proteome protein microarray has been used to screen antibodies for their specificity and cross-reactivity (Michaud et al., 2003). Lueking et al. (2003) generated a glass chip-based high-density protein microarray from 2413 non-redundant purified human fusion proteins on a polymer surface at a spot density of 1600 proteins/cm². These protein microarrays were used to characterise antibody binding, specificity and cross-reactivity. Testing the cross-reactivity of antibodies or recombinant capture molecules is a prerequisite for their application in highly multiplex antibody array assays. Recently, various functional activities of 50 SNP variants of the tumour suppressor gene p53 were analysed (Boutell et al., 2004). The p53 variants were analysed for DNA binding ability, the capability of being phosphorylated and the binding of ubiquitin ligase MDM2 in parallel. Functional differences of the p53 oncogene were shown in relation to the mutation within the p53 oncogene. Such an approach might help explain the different effects of p53 mutations during cancer progression.

Protein interaction studies are needed in order to study the network of proteins, but give no information about the interaction sites. Protein domain microarrays address this question. The protein domains serve as modules of recognition for the assembly of multiprotein complexes. A protein-domain microarray, containing GST-fusion proteins with peptide-specific binding motifs such as SH3, SH2 (Src homology 2 and 3) or PDZ domains (a domain originally identified in PSD-95, DLG and ZO-1 proteins), was established by Espejo et al. (2002). These researchers were able to confirm the domain binding profile of the signalling molecule Sam68. Furthermore, a new binding profile for the core small nuclear ribonucleoprotein, SmB' was discovered. With the same approach it was also possible to detect an overlapping recognition site of 14-3-3 and Akt for the tumour suppressor tuberlin (Liu et al., 2002).

Another domain–domain interaction study was done by Newman and Keating (2003). Forty-nine coiled strands of human basic-region leucine zipper (bZIP) transcription factors were screened against each other in a peptide array (Newman and Keating, 2003). Sixteen bZIP families showed 136 potential inter- and intrafamily interactions. Most of the identified interactions could be verified in both directions. In total, 50 of 58 known interactions were confirmed. The data obtained from the protein, protein-domain and peptide microarrays will contribute to our understanding of complex protein–protein interactions in cell signalling processes.

Another aspect of analysing protein function is the study of their enzymatic activity. Miniaturised multiplexed assay systems are well suited for measuring kinases, proteolytic activity and specificity within a single experiment (Houseman et al., 2002; Reineke et al., 2001; Winssinger et al., 2002; Zhu et al., 2000). These kinds of enzyme–substrate arrays have been described by different groups using recombinant proteins or peptides immobilised onto solid supports. In order to measure kinase activity, phosphorylation was detected either by radioisotopes [$\gamma^{32}\text{P}$] ATP labelling, phospho-aminoacid selective antibodies or by phospho-peptide specific fluorescent dyes (Houseman et al., 2002; Martin et al., 2003; Reineke et al., 2001; Zhu et al., 2000). Zhu and colleagues cloned and arrayed 119 of the 122 known or suspected yeast kinases. Seventeen different substrates were screened using a radioactive kinase assay. Thus they could identify novel activities of some kinases. As an alternative for radioactive detection of kinase activity and phosphorylated proteins on microarrays the applicability of a fluorescent dye that is specific for phosphorylated amino acid side chains has been described (Martin et al., 2003).

Protein interaction arrays may also be integrated into drug screening processes. Such an approach relies on immobilised small organic compounds in order to screen for receptor–ligand interactions. First results indicated that this technology enables parallel high-throughput screening for ligand–receptor interactions with very small quantities of small molecule ligands and target proteins. So far however, this approach has not been established in a high-throughput fashion (MacBeath et al., 1999; Winssinger et al., 2002). An alternative approach was developed by Gosalia and Diamond (2003). Chemical libraries were printed on microarrays in order to perform fluid phase nanoliter reactions. The chemical compounds were deposited in a microarray format within individual nanoliter droplets of glycerol. Following reagents were added by aerosol deposition onto the glass slides. This approach allowed the rapid addition of multicomponent reactions without cross-contamination or the usage of bordering cavities. The feasibility was demonstrated using kinetic profiling of protease mixtures and protease–substrate interactions. An inhibitor of caspases 2, 4 and 6 was identified using a 352-compound combinatorial library microarray. This technol-

ogy might be of interest in accelerating screening procedures for active substances.

4. Outlook

The potential of protein microarrays has still not been fully exploited, although the principles of protein microarray technology were already described and established years ago. An increasing number of applications have already demonstrated the extraordinary power of protein microarray technology. Multiplex assays enable the identification and quantification of proteins and the study of protein interactions with other proteins, peptides, low molecular weight compounds, oligosaccharides or DNA. Proteomic research, high-throughput drug compound screening and diagnostic applications call for multiplex protein analysis, which can currently not be fulfilled by 2D PAGE/MS analysis. Protein microarrays will no doubt accelerate protein expression profiling and protein–protein interaction analysis.

The limiting factors of protein microarray technology are well characterised. The desired high number of functionally active recombinant proteins and capture molecules with high affinity, specificity and selectivity is still lacking. High-throughput methods for protein expression and capture molecule generation are applied in order to address this content problem. The fabrication of high-density arrays demands a tremendous effort, not only in generating appropriate capture molecules like monoclonal antibodies, recombinant scaffolds or aptamers. Before it becomes possible to apply protein microarrays in high-throughput screening approaches or in clinical diagnostics, they have to demonstrate precision, sensitivity and reliability in fully automated systems. At present, HTS systems used by the pharmaceutical industry or immunoassays used for clinical diagnostics are highly automated and extremely robust. Any new assay format has to compete with this highly sophisticated technology not only with regard to its performance, but also with regard to its costs. New instruments in combination with novel assay formats always involve enormous investments. As long as only a few additional analytes have to be detected from the same sample, microarrays are not competitive. In such a scenario, it will be much more economical to increase the throughput of the currently available automated systems. However, with an increasing number of analytes (as they are already available in allergy diagnostics, for example) and the simultaneous screening of potential drug candidates for selectivity and cross-reactivity, the multiplexing approach becomes a very promising alternative. The reduction of sample volume is of great importance for all those applications where only minimal amounts of samples are available (e.g. analysis of multiple tumour markers from a minimum amount of biopsy material). Therefore, protein microarray-based technology will be successfully applied to different aspects within the broad field of proteomics and diagnostics.

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